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NTP Designated Federal Official
Office of Liaison, Policy and Review
National Toxicology Program
National Institute for Environmental Health and Safety
PO Box 12233, MD K2-03
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Re: Comment on Draft Technical Report 582 and Request for Postponement of Peer Review

Dear Dr. White,

The National Toxicology Program (NTP) recently announced the availability of several draft technical reports of carcinogenicity bioassays, including studies of vinylidene chloride (TR 582), for public comment and peer review. 78 Fed. Reg. 54476 (September 4, 2013). The notice indicated that the draft reports would be available September 20, the comment deadline would be October 15, and the peer review meeting would be October 29. These comments are submitted on behalf of the producers of vinylidene chloride. For the reasons discussed below, we respectfully request postponement of the peer review.

On October 1, shortly after the reports became available, nonessential functions of the federal government shut down. As a result, there has been no one available at NTP with whom we could speak to obtain individual animal data, and no pathologists available with whom to discuss our concerns. Twenty-five days would have been a short period in which to review the draft report and formulate meaningful comments in any event, but with no one available to provide us the needed data the difficult has become impossible. Given the issues created by the staff furloughs it may be that NTP has already decided to postpone the peer review, but if not we urge that you give this request serious consideration. Our interim comments, which support this request, follow.

Draft TR 582 states that the vinylidene chloride bioassays demonstrate clear evidence of carcinogenicity in male rats, male mice and female mice, and some evidence of carcinogenic activity in female rats. These results are inconsistent with those from *eleven* previous cancer bioassays on this substance, although it is recognized that some of these suffered from methodological deficiencies. The potential significance of these bioassays, the absence of positive findings in previous studies, and the very short time given to review the draft report all support our request that the peer review of this report be delayed and more

information provided to reviewers to allow a more robust assessment of the findings and their relevance to the hazard characterization of vinylidene chloride. Failing that, we believe the results should be considered no more than equivocal.

Maximum Tolerated Dose

Our review of the NTP cancer bioassays in rats and mice reveals that the maximum tolerated dose (MTD) was clearly exceeded at all tested dose levels, based upon Environmental Protection Agency (EPA) Health Effects Division (HED) Guidance for dose level selection of carcinogenicity studies [1]. Therefore, while the data in these cancer bioassays are interesting from a scientific point of view, they cannot be used for human health risk assessment, classification and labelling, or any other scenario for assessment of potential human health concerns. We would also like to remind NTP that the conduct of assays that fail to adhere to the appropriate guidance, particularly when doing so results in findings that could be considered as of limited to no relevance to humans, may constitute a waste of experimental animals, something that all toxicologists should strive to avoid.

As an example, the dose levels for the rat study were set based on the 2-week and 13-week studies. The rationale for the top dose being the MTD was based on an overall assessment of toxicity including mortality and body weight loss. Based on body weights in the 90-day study, both sexes of mice clearly significantly exceeded MTD. The EPA HED Guidance *defines* MTD as equal to a body weight gain of 10% (note that MTD is defined as change in body weight *gain* vs. just change in body weight). Thus for mice, even the 6.25 ppm dose in the female mouse 90-day study resulted in a body weight gain depression of 27%, and was 39% in females at 25 ppm (6.25 and 25 ppm were the low and high doses selected for the bioassay). Thus, based on body weight gain depression, all female doses exceeded MTD. Interestingly, the dose separation between high and low dose for mice was only 4x, also highly unusual (10 is norm). For male mice, body weight gain depression was 24% at 25 ppm, also clearly exceeding MTD. For rats, 90-day body weight gain depressions were insignificant, so use of the "necrosis" is indeed the correct indicator of exceeding MTD.

NTP scientists have noted that it is desirable to use a "minimally toxic dose" for dose selection for chronic bioassays, and that severe body weight loss is a disqualifier ("Moreover, slight body weight reductions may be the 'minimal toxicity' that is expected at a properly chosen MTD") [2]. Further, NTP's own leadership has recognized that "[A]cceptable lesions in the nasal cavity included minimal to mild hyperplasia, metaplasia of the squamous and/or respiratory epithelium, olfactory degeneration, and/or minimal to mild inflammation. *Unacceptable lesions included necrosis.* . . ." [3] Likewise, EPA's Guidelines for Carcinogen Risk Assessment specifically point to the need for caution in interpreting tumor data when the MTD is exceeded, and particularly when considered in the context of "other study results and other lines of evidence" [4]. Eight other negative bioassays would appear to be an important contextural other line of evidence.

Given the findings in the respiratory tract of the rats in the 13-week study and the 2-year bioassay one can argue that even at the lowest dose there was sufficient evidence of toxicity to conclude that the MTD was exceeded: "A combination of lesions in the nasal

epithelium composed of olfactory epithelium atrophy, mineralization, and *necrosis* and turbinate atrophy was observed with generally increasing severity with increasing exposure to vinylidene chloride." "Olfactory epithelial necrosis occurred at the dorsal meatus, dorsal septum, and all regions of ethmoturbinates in Level III of the nose. Necrosis of the olfactory epithelium was characterized by areas of nuclear pyknosis of the epithelium, fragmentation, and hypereosinophilia, and in some areas, *full-thickness sloughing of the epithelium and cell debris* into the nasal passages at Level III." This was noticed at all doses, suggesting that MTD was achieved from doses at and above 12.5 ppm.

The EPA HED Guidance on MTD in cancer bioassays states that: "Single cell/focal necrosis, if observed at the same dose level in 5-10% of the animals in multiple prechronic/chronic studies, is sufficient evidence that a dose is adequate." From the description of the findings, it appears that necrosis was broadly apparent across multiple sites in the nasal tract. If these lesions were not focal in nature, there is additional reason to believe that MTD was exceeded. If, however, there was a dose-dependent transition from focal necrosis to widespread necrosis, this would indicate MTD may have been exceeded only at the higher doses. Since all exposed rats in the 13-week study demonstrated clear signs of focal necrosis at dose levels of >12.5ppm, it is clear that the MTD was exceeded by even the lowest dose group of 25 ppm, bringing into question the relevance of the tumor findings in this study.[1]

In order to perform a thorough assessment of the impact of exceeding the MTD a review of the individual animal data would be necessary, along with a discussion with the study pathologist. In any event, we question how it could be considered appropriate to run a study with such tight spacing so close to the LD_{50}, with no apparent intention of determining a NOAEL based upon dose level selection.

In addition to this serious concern, we offer the following specific comments on the draft report that need to be addressed.

Genotoxicity

The summary of genotoxicity data provided within the report provides an inconsistent and incorrect description of the genotoxic potential of vinylidene chloride. A paragraph in the discussion and conclusions section states that the genotoxic potential of vinylidene chloride is demonstrated, but this conclusion cannot be substantiated by the data in the report and it is not in line with other recent assessments based on approximately the same data set. The lack of convincing *in vivo* genotoxicity of vinylidene chloride is not adequately addressed in the draft report and further work is required to interpret the findings in the 2-year assays when considering the lack of a NOAEL for non-neoplastic findings in the 2-year study and the lack of detectable *in vivo* genotoxicity.

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[1] The NTP incidence table for necrosis indicates that 10/10 rats male rats exhibited necrosis at 12.5 ppm, and 6/10 female rats at 25 ppm. Thus, the necrosis indicator of MTD is exceeded at 12.5 ppm (males) and 25 ppm (females). The conclusion is inescapable that even the lowest dose of the rat bioassay (25 ppm) exceeded MTD. Again, we question the design of a bioassay with only a 4x difference between high and low dose (25 ppm and 100 ppm).

Four genotoxicity studies are cited in the abstract (page 13), a negative Ames assay according to Mortelmans *et al.* [2], a positive mammalian mutagenicity assay according to McGregor *et al.* [3], a negative sex-linked recessive lethal mutation assay according to Foureman *et al.* [4], and a negative micronucleus assay *in vivo* according to MacGregor *et al.* [5]. The abstract draws no general conclusion from these four studies about the genotoxic potential of vinylidene chloride. In the results section (page 108), the same four studies are cited, again without a conclusion about the genotoxic potential of vinylidene chloride. In Appendix E of the draft report, brief descriptions of the four study protocols and the corresponding results are provided in tabulated format. In the discussion and conclusions section (page 121), however, the paragraph on genotoxicity testing begins: "*The results from a variety of genetic toxicology studies, including approaches such as bacterial mutagenicity assays, yeast test systems, mammalian cell lines, and in vivo tests indicate that vinylidene chloride has mutagenic, clastogenic, and aneugenic properties.*" This statement is inconsistent with the information provided in the report (three negative studies and one positive study), particularly for the clastogenic and aneugenic properties which are not confirmed in the *in vivo* micronucleus assay. In addition, the negative sex-linked recessive lethal mutation assay which detects the occurrence of mutations (point mutations and small deletions) did not confirm the mutagenic properties of vinylidene chloride observed in the *in vitro* mammalian mutagenicity assay.

The draft report fails to mention several significant weaknesses of the only positive study cited, the mouse lymphoma mutagenicity study. According to extensive reviews by Kirkland *et al.* [6] and Mathews *et al.* [7] of the standard *in vitro* genotoxicity tests, a positive result in the mouse lymphoma mutagenicity test only should not be regarded as conclusive, considering the assay's specificity of 39.0% and 57.8% respectively. Moreover the test used the L5178Y cell line which has a dysfunctional p53 protein and as such is abnormally susceptible to the induction of genetic alterations [8][9]. In addition, the mouse lymphoma mutagenicity test as reported by McGregor *et al.* [3] was specifically designed to deal with gases and volatile liquids, but since the doses were only described as relative volumes (% in 250 ml flask containing 10 ml medium) the actual concentrations of vinylidene chloride (water solubility of 2.5 g/l [9]) to which the cells were exposed are difficult to trace back. A 0.16% fraction of 250 ml being saturated vinylidene vapor represents approximately 10 mg to be distributed between an air phase and 10 ml medium (vinylidene chloride vapor pressure: 66.3 kPa [10]). The mouse lymphoma mutagenicity test was conducted under conditions at which cytotoxicity was well controlled, but it remains difficult to assess at which concentrations the L5178Y cells were exposed and how these concentrations relate to the maximum concentration levels as defined in the OECD and ICH guidelines [11][12]. All these aspects hamper the interpretability of the study when characterizing the genotoxic potential of vinylidene chloride.

The discussion and conclusions section cites an additional study in support of the assumed genotoxic potential of vinylidene chloride, a study by Reitz *et al.* [13] whose results are interpreted as confirming the alkylating potential of vinylidene chloride. Indeed nucleotide alkylation was detected, but this was judged by the authors of the manuscript as "*extremely low.*" The highest level of alkylation was observed in the kidneys of mice exposed to 50 ppm and corresponded to 0.003%. This level was significantly lower than the 0.3-0.4%

observed for the potent *in vivo* alkylating agent dimethylnitrosamine [14]. Moreover Reitz *et al.* demonstrated that the significant cellular damage induced by vinylidene chloride exposure (50 ppm) was followed by significant DNA-replication (tissue repair) and only limited DNA-repair, whereas dimethylnitrosamine resulted in little DNA-replication and significant DNA-repair. In contrast to the interpretation in the draft report, Reitz *et al.* concluded that "*Since the observation of DNA repair implies that some sort of previous alteration to DNA has taken place, the low potency of vinylidene chloride in this system again suggests that its in vivo potential for producing mutagenic effects is much less than that of dimethylnitrosamine. The failure to demonstrate significant genetic effects with tumorigenic doses of vinylidene chloride suggested that epigenetic mechanisms might be operating.*" In addition, there is increasing evidence that DNA alkylation should be interpreted as a marker of exposure rather than a marker of effect [15].

Other assessments of the genotoxic potential of vinylidene chloride have been recently performed. A CICAD report on vinylidene chloride published in 2003 [16] states that there is a fairly extensive database on its genotoxicity. While vinylidene chloride was found to cause gene mutations in microorganisms, the report concluded that most tests with mammalian cells show no evidence of genetic toxicity, and that the test battery is incomplete because it lacks a test for chromosomal damage in the mouse lymphoma. This conclusion suggests that the positive result reported by McGregor *et al.* which is mentioned in the draft report was not considered of sufficient validity to meet the requirements. The European Scientific Committee on Occupational Exposure Limits (SCOEL) adopted this conclusion in 2007 [17].

Moreover, a complete re-assessment of genotoxic potential was performed for the REACH dossier of vinylidene chloride. This assessment concluded that overall, vinylidene chloride appeared to show some genotoxic activity in *in vitro* testing systems, especially in the presence of metabolic activation. However, based on the absence of positive findings in (i) a micronucleus test conducted in mouse bone marrow and in mouse fetal erythrocytes [18], (ii) a dominant lethal test with male CD rats [19], and (iii) a micronucleus study in Sprague Dawley rats after chronic exposure to vinylidene chloride [20], the substance was not considered genotoxic *in vivo*. The negative studies reported by MacGregor *et al.* [5] and Foureman *et al.* [4] which are cited in the draft report corroborate the conclusion in the REACH dossier.

Considering that a genotoxic mode of action of vinylidene chloride as the explanation for the findings in the 2-year studies cannot be substantiated by the results of the available *in vivo* genotoxicity assays, possible non-genotoxic modes of action should be addressed in greater detail because the mode of action will have significant influence on any risk assessment based on the results of the study. The assessment of possible non-genotoxic modes of action will require an additional in-depth assessment of the non-neoplastic findings in both the 3-month (not provided in the draft Technical Report) and 2-year studies. In addition, the 2-year studies do not include a dose level which can be identified as a NOAEL; this lack might complicate the identification of the adverse outcome pathway(s) and the differentiation between direct or indirect modes of action, with the subsequent determination of a threshold on which the risk assessment and classification analysis should be based (*i.e.*,

the determination of whether the presence of non-neoplastic lesions is a prerequisite for the appearance of neoplastic lesions).

In these circumstances, the genotoxicity findings are "equivocal" at best.

Comparison of Tumor Findings with Historical Control Data

The significance of some tumor types is demonstrated via comparison with historical control data, however in some cases (*e.g.,* the female rat thyroid tumours and leukemias) the study tumor incidence is compared only to historical controls from inhalation studies, whereas for other tumor types (*e.g.,* the female mouse hepatocholangiosarcomas) the incidence in the study is compared to historical controls from all routes of exposure. The discussion should be consistent in how the historical control data are used.

Mode of Action for Kidney Tumors in Mice

The discussion of the potential mode of action for kidney tumors in mice is inappropriate, specifically with respect to comparison to trichloroethylene. The draft report indicates that, like trichloroethylene, glutathione conjugation in the liver followed by subsequent metabolism in the kidney via the beta-lyase pathway to form reactive metabolites could be responsible for the kidney toxicity observed in male mice and the increase in tumor incidence. There is no evidence that this occurs with vinylidine chloride, and this should be clearly stated so that the reviewer is not misled.

It should also be noted that in the case of trichloroethylene renal tumors (very low incidence) are observed in rats but not mice. Therefore the existence of the glutathione/beta-lyase pathway in mice does not lead to kidney carcinogenesis of trichloroethylene in mice at doses far higher than those causing effects in rats. It is therefore inappropriate to state that this pathway could be responsible for producing mouse kidney tumours in the case of vinylidine chloride without actual mechanistic data to support this assumption.

Respectfully submitted,

[redacted]

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